Saskatchewan
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AGRICULTURE DEVELOPMENT FUND

FINAL REPORT

20020057

AN INTERNATIONAL COLLABORATION FOR THE DEVELOPMENT OF NEW AND IMPROVED SOURCES OF ASCOCHYTA RESISTANCE IN CHICKPEA

Funded by: The Agriculture Development Fund

September, 2006

Prepared by: University of Saskatchewan

2007-04719/

An International Collaboration for the Development of New and Improved Sources of *Ascochyta* Resistance in Chickpea

Final Report on Project No. SAFRR2002005781D

June 30th 2006

M. Lulsdorf, B. Tar'an, T. Warkentin, B. Vandenberg

Crop Development Centre
University of Saskatchewan
51 Campus Drive
Saskatoon, Sk. S7N 5A8

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A. ABSTRACT

In collaboration with researchers from India and Australia, this project investigated the development of interspecific hybridization technology for chickpea in order to access effective sources of ascochyta blight resistance. *C. bijugum* and *C. judaicum* accessions that showed improved blight resistance during the early growth stages were selected and crossed with the cultigen. Confirmed *C. arietinum* x *C. bijugum* hybrids were obtained, but all subsequent rooting protocols failed. Hybrids could not be induced to flower, and thus back-crossing to the cultivated chickpea was not accomplished. Improvements to the interspecific hybridization protocol are necessary if wild *Cicer* species are to be utilized as sources of blight resistance.

B. EXECUTIVE SUMMARY

Resistance to the aggressive *Ascochyta rabiei* strains found in the soils of western Canada is lacking in our current chickpea cultivars. Therefore, new sources of ascochyta blight resistance are urgently needed. Wild related *Cicer* species are valuable sources of resistance to many pest and diseases. This project involved the development of interspecific hybridization technology for chickpea in order to access new and effective sources of ascochyta blight resistance. The work was carried out in close collaboration with Dr. Heather Clarke (CLIMA, Australia) and Dr. Nalini Mallikarjuna (ICRISAT, India).

Wild related annual *Cicer* species were tested in the field and in the greenhouse to identify accessions with improved blight resistance (see Project # 20020160-04AC for details). *C. bijugum* ILWC 260 and *C. judaicum* accessions ILWC 164 and 165, were identified as having improved resistance levels during the early growth stages. These accessions were selected and improvements to the interspecific hybridization protocol were specifically targeted to produce hybrids with these two species. Results showed that hybrid ovules needed rescue no later than 10 - 12 days after pollination and the medium composition needed to be modified due to the very young growth stage of the embryos at the time of rescue. Improvements to the vitamin composition, organic nitrogen and carbohydrate levels were also made. To date, two confirmed *C. arietinum* x *C. bijugum* and several putative hybrids of *C. arietinum x C. judaicum* have been recovered.

Hybrid shoots were micro-propagated in order to conduct rooting experiments. To date, very few hybrid shoots could be rooted due to their lack of vigour regardless if rooting media or grafting protocols were used.

The following bridge crosses were made: *C. arietinum* x *C. echinospermum* and *C. arietinum* x *C. pinnatifidum*. Offspring from these crosses were then back-crossed with *C. bijugum* or *C. judaicum*. In addition, crosses were also made between *C.*

pinnatifidum x *C. judacium* and subsequently back-crossed with *C. arietinum*. However, no viable embryos/plants could be obtained from any of the bridge-crosses.

Over 6,000 crosses were made between the cultivated chickpea and wild accessions of *C. bijugum* and *C. judaicum* over a 1½ year period. Yet few viable hybrids were obtained and subsequent hybrid growth was poor. Hence, even though hybridization barriers were not at the pre-fertilization stage, there were clearly post-fertilization barriers such as embryo abortion, lack of endosperm initiation and/or premature endosperm breakdown. Improvements to the interspecific hybridization protocol are necessary if wild annual *Cicer* species are to be utilized as sources for ascochyta blight resistance.

C. ABBREVIATIONS

ATC = Australian Temperate Crops Collection, Horsham, Victoria
CLIMA = Centre for Legumes in Mediterranean Agriculture, Crawley, Australia
ICARDA = International Centre for Agricultural Research in the Dry Areas, Aleppo, Syria
ICRISAT = International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India
USDA = National Genetic Resources Program, Pullman WA, USA

DAP = days after pollination **BAP** = 6-benzylaminopurine; **2,4-D** = 2,4-dichlorophenoxy acetic acid; **GA_3** = gibberellic acid; **IAA** = 3-indole acetic acid; **IBA** = indole butrytic acid; **NAA** = 1-naphthalene acetic acid; **PEG** = polyethylene glycol; **PVP** = polyvinyl polypyrrolidone

D. TECHNICAL REPORT

1. Project Background

Ascochyta blight is one of the major constraints to chickpea production in western Canada and contributed to a drastic reduction in acreage in 2002-2004. Disease outbreaks of this fungus cause reduced yields, poor seed quality and increased production costs (Pearse 2001). High levels of post-flowering resistance to this fungus are lacking in our current cultivars (Warkentin *et al* 2001), and Saskatchewan could face further substantial economic losses in the future. Furthermore, recent research has shown that there has been a shift in the ascochyta blight (*A. rabiei*) population to more aggressive strains suggesting that the disease severity will likely increase in the future (Banniza *et al* 2004). Therefore, new sources of ascochyta blight resistance are urgently needed.

Wild related *Cicer* species possess a wealth of genes not only for ascochyta blight resistance but also for many other agronomically important traits such as fusarium wilt resistance, cold and drought tolerance. Transferring genes from wild related to the

cultivated species through interspecific hybridization technology has been done in many species such as bean, lentil, barley, and wheat. Hence, having access to this germplasm resource will open the genetic bottleneck present in this species and consequently will have a long-term beneficial impact on Canadian chickpea breeding.

2. Project Objectives

The objective of this project was to develop interspecific hybridization technology for chickpea in order to access new and effective sources of ascochyta resistance in collaboration with researchers from CLIMA, ICARDA and ICRISAT. Project goals were as follows:

- Acquire wild *Cicer* accessions
- Increase seeds from wild accessions and maintain in long term storage
- Test accessions for *Ascochyta rabiei* resistance (in collaboration with project # 20020160-04AC)
- Develop interspecific hybridization protocol
- Cross selected wild species with cultivated chickpea
- Recover interspecific hybrids and self or back-cross

For the third year, the following milestones were identified for this project:

- Use of bridging species, if wild accessions are not directly cross-able
- Morphological and genetic authentication of hybrids
- Improvements to rooting protocol of hybrids and adaptation to *ex vitro* conditions
- Pollen fertility assessment and cytogenetic characterization of hybrids
- Selfing or back-crossing of hybrids and determination of problem areas
- Transfer of hybrid material and/or selfed or back-crossed hybrids to the CDC pulse breeding program for cultivar improvement

3. Results and Discussion

3.1. *Cicer* acquisitions and seed increase

Chickpea (*Cicer arietinum* L.) belong to the genus *Cicer*, section Monocicer (van der Maesen 1987). This species probably originated in Turkey, Syria and surrounding areas. *C. reticulatum* is considered the wild progenitor and is readily cross-able with the cultigen (Ladizinsky 1975). *C. echinospermum* belongs to the secondary gene pool and can be crossed with chickpea cultivars but sterility can be a problem. *C. pinnatifidum*, *C. bijugum*, *C. judaicum* belong to the tertiary gene pool and to date, only *C. pinnatifidum* has been successfully crossed with the cultivated chickpea. The other annual chickpea species *C. cunneatum*, *C. chorassanicum* and *C. yamashitae* are more distantly related (Van der Maesen 1987, Ahmad *et al* 2005).

Table 1. *Cicer* accessions and *Ascochyta* blight rating (R or 1 = resistant; MR or 5 = moderately resistant; S or 9 = susceptible)

Cicer Species	ICARDA No.	USDA PI No.	ICRISAT No.	Selection From	*Ascochyta* Rating Literature[1]	*Ascochyta* Early Growth Mean	Rating Late Stages ± Std Error	Rating Class
Bijugum	ILWC 7				2, 4		6.0 ±0.29	S
Bijugum	ILWC 8	458552			3, 7		6.2 ±0.86	S
Bijugum	ILWC 34	458550			3, 4, 6		6.4 ±0.46	S
Bijugum	ILWC 62	458551		ILWC 7	3, 4		8.5 ±0.38	S
Bijugum	ILWC 63			ILWC 7	2, 4		8.5 ±0.38	S
Bijugum	ILWC 64			ILWC 7	2, 5		8.6 ±0.25	S
Bijugum	ILWC 65			ILWC 7	3, 5		9.0 ±0.00	S
Bijugum	ILWC 66			ILWC 7	3, 5		8.0 ±0.00	S
Bijugum	ILWC 67			ILWC 7	3, 6		8.6 ±0.25	S
Bijugum	ILWC 68			ILWC 7	3, 6	4.7 ±1.55	9.0 ±0.00	S
Bijugum	ILWC 69			ILWC 7	3	4.9 ±2.15	8.0 ±0.00	S
Bijugum	ILWC 70			ILWC 7	3		7.7 ±0.33	S
Bijugum	ILWC 71			ILWC 7	3, 6		8.6 ±0.25	S
Bijugum	ILWC 72			ILWC 7	4, 5		9.0 ±0.00	S
Bijugum	ILWC 73			ILWC 7	3		7.3 ±0.33	S
Bijugum	ILWC 74			ILWC 7	2,5		7.7 ±0.33	S
Bijugum	ILWC 75			ILWC 7	2, 5, 6		7.2 ±0.40	S
Bijugum	ILWC 76			ILWC 7	1, 2, 5, 6	5.7 ±0.44	8.5 ±0.50	S
Bijugum	ILWC 77			ILWC 7	3		7.2 ±0.40	S
Bijugum	ILWC 177	458550		ILWC 34	3, 6	5.0 ±0.66	8.0 ±0.00	S
Bijugum	ILWC 178	458550		ILWC 34	5, 6		8.6 ±0.25	S
Bijugum	ILWC 194			ILWC 42	4, 7		8.0 ±0.00	S
Bijugum	ILWC 209	599065			7		9.0 ±0.00	S
Bijugum	ILWC 217		ICC 17122	ICCW 7	2, 5		9.0 ±0.00	S
Bijugum	ILWC 220	488552	ICC 17125	ICCW 10	4, 6	4.5 ±0.41	7.9 ±0.41	S
Bijugum	ILWC 227		ICC 17156	ICCW 41	4, 6		7.8 ±0.48	S
Bijugum	ILWC 228	599037		ICCW 42	3, 6		7.8 ±0.22	S
Bijugum	ILWC 240	599062			2, 6	5.3 ±0.31	7.8 ±0.24	S
Bijugum	ILWC 241	599063			2, 9	5.0 ±0.00	7.5 ±0.68	S
Bijugum	ILWC 260				2, 3	3.7 ±0.41	6.6 ±1.06	S
Bijugum		599065					8.4 ±0.47	S
Bijugum		599066					8.9 ±0.23	S
Chorassanicum	ILWC 16	458553	ICC 17141	ICCW 26			9.0 ±0.00	S
Cunneatum	ILWC 19	458554		ICCW 47			7.8 ±0.44	S
Cunneatum	ILWC 232		ICC 17162	ICCW 47	7		8.0 ±0.00	S
Echinospermum	ILWC 179			ILWC 35	6		8.5 ±0.50	S
Echinospermum	ILWC 238	599068					8.0 ±0.00	S
Echinospermum	ILWC 245	527930			2, 5		8.5 ±0.29	S
Echinospermum	ILWC 246	527932			5		6.5 ±0.38	S
Echinospermum		489776		ICCW 44	6		8.5 ±0.50	S
Echinospermum		599040					6.0 ±0.00	MR
Echinospermum		599041					8.3 ±0.25	S
Judaicum	ILWC 20	510659	ICCW 34				6.8 ±0.80	S
Judaicum	ILWC 30	458560	ICCW 36				6.4 ±1.23	S
Judaicum	ILWC 44	599055					6.4 ±0.66	S
Judaicum	ILWC 45	599056					6.3 ±0.21	S
Judaicum	ILWC 58			ILWC 4			6.8 ±0.32	S
Judaicum	ILWC 59			ILWC 4	R		6.3 ±0.33	S

Cicer Species	ICARDA No.	USDA PI No.	ICRISAT No.	Selection From	*Ascochyta* Rating Literature[1]	*Ascochyta* Early Growth Mean	Rating Late Stages ± Std Error	Rating Class
Judaicum	ILWC 161			ILWC 30			6.5 ±0.29	S
Judaicum	ILWC 163			ILWC 31			5.8 ±0.49	MR
Judaicum	ILWC 164			ILWC 31		4.3 ±2.36	6.0 ±0.00	MR
Judaicum	ILWC 165			ILWC 31		3.2 ±2.62	5.8 ±0.48	MR
Judaicum	ILWC 166			ILWC 31		4.0 ±0.00	6.0 ±0.71	MR
Judaicum	ILWC 167			ILWC 31		3.5 ±0.50	5.8 ±0.49	MR
Judaicum	ILWC 168			ILWC 33			8.0 ±0.32	S
Judaicum	ILWC 173			ILWC 33			8.0 ±0.00	S
Judaicum	ILWC 174			ILWC 33			8.8 ±0.20	S
Judaicum	ILWC 175			ILWC 33			7.0 ±0.41	S
Judaicum	ILWC 176			ILWC 33			7.0 ±0.32	S
Judaicum	ILWC 198			ILWC 47		3.3 ±0.00	5.3 ±0.75	MR
Judaicum	ILWC 223		ICC 17148	ICCW 33			6.4 ±0.40	S
Judaicum	ILWC 255	599077			2		5.2 ±0.58	MR
Judaicum		458559	ICC 17150	ICCW 35			6.3 ±0.33	S
Judaicum			ICC 17192	ICCW 77			6.2 ±0.37	S
Judaicum			ICC 17196	ICCW 81		3.0 ±0.00	In Progress	
Judaicum			ICC 17199	ICCW 85			6.2 ±0.37	S
Pinnatifidum	ILWC 19	510663					7.6 ±0.93	S
Pinnatifidum	ILWC 22	458557		ILWC 9			7.3 ±1.11	S
Pinnatifidum	ILWC 51	599060					8.0 ±0.00	S
Pinnatifidum	ILWC 60						8.8 ±0.20	S
Pinnatifidum	ILWC 159						6.3 ±0.85	S
Pinnatifidum	ILWC 160						8.7 ±0.33	S
Pinnatifidum	ILWC 162						8.0 ±0.32	S
Pinnatifidum	ILWC 169			ILWC 9		4.0 ±0.00	6.0 ±0.84	MR
Pinnatifidum	ILWC 170			ILWC 9			8.3 ±0.48	S
Pinnatifidum	ILWC 171			ILWC 9	5		6.8 ±0.37	S
Pinnatifidum	ILWC 172			ILWC 9			8.7 ±0.33	S
Pinnatifidum	ILWC 212	599070					9.0 ±0.00	S
Pinnatifidum	ILWC 224	458555	ICC 17152	ICCW 37			8.2 ±0.37	S
Pinnatifidum	ILWC 226		ICC 17155	ICCW 40			6.8 ±0.25	S
Pinnatifidum	ILWC 236	593716					8.7 ±0.40	S
Pinnatifidum	ILWC 249	518861					8.3 ±0.25	S
Pinnatifidum	ILWC 250	518862			2, 4		8.0 ±0.32	S
Pinnatifidum	ILWC 251	518863			2		7.0 ±0.58	S
Pinnatifidum	ILWC 252			ILWC 226			8.4 ±0.25	S
Pinnatifidum			ICC 17200	ICCW 85			7.0 ±0.55	S
Pinnatifidum		458556		ICCW 38			7.0 ±1.15	S
Reticulatum	ILWC 118			ILWC 21	5		6.5 ±0.56	S
Reticulatum	ILWC 120			ILWC 21			7.0 ±1.00	S
Reticulatum	ILWC 122			ILWC 21	7		6.0 ±0.00	MR
Reticulatum	ILWC 123			ILWC 21	6		5.0 ±0.00	MR
Reticulatum	ILWC 124			ILWC 21	7		In Progress	
Reticulatum	ILWC 125			ILWC 21	8		8.0 ±0.00	S
Reticulatum	ILWC 126			ILWC 21	7		In Progress	
Reticulatum	ILWC 127			ILWC 21			7.5 ±0.50	S
Reticulatum	ILWC 129			ILWC 21	6		7.0 ±0.98	S
Reticulatum	ILWC 130			ILWC 21	5		7.0 ±0.00	S
Reticulatum	ILWC 132			ILWC 21	6		7.8 ±0.25	S
Reticulatum	ILWC 133			ILWC 21			6.5 ±0.50	S
Reticulatum	ILWC 134			ILWC 21	6	4.0 ±0.00	6.5 ±0.38	S
Reticulatum	ILWC 135			ILWC 21	6	5.0 ±0.00	6.3 ±0.50	S
Reticulatum	ILWC 136			ILWC 21	7	4.0 ±0.00	6.5 ±0.50	S

Cicer Species	ICARDA No.	USDA PI No.	ICRISAT No.	Selection From	*Ascochyta* Rating Literature[1]	*Ascochyta* Early Growth Mean	Rating Late Stages ± Std Error	Rating Class
Reticulatum	ILWC 137			ILWC 21	6		7.5 ±0.50	S
Reticulatum	ILWC 247	527934					7.0 ±0.00	S
Reticulatum	ILWC 253	510655		ILWC 216	8		In Progress	S
Reticulatum	ILWC 254	510656		ILWC 218			6.7 ±0.33	S
Reticulatum		572537					7.5 ±0.50	S
Reticulatum	ILWC 242	593709			7		8.3 ±0.33	S
Yamashitae	ILWC 214	510657	ICC17116	ICCW 1			8.3 ±0.43	S
Yamashitae	ILWC 215	504550	ICC17117	ICCW 2			8.5 ±0.43	S

[1]Collard *et al* 2001, Nguyen *et al* 2005, Robertson *et al* 1995, Singh *et al* 1998

Wild *Cicer* species accessions listed in Table 1 were obtained from ICARDA, ICRISAT, USDA, CLIMA/ATC and Dr. Faiz Ahmad (Brandon, Manitoba). A total of 110 *Cicer* accessions were screened in this project representing all 9 annual wild *Cicer* species. Thirty two of the accessions were classified as *C. bijugum*, 1 was *C. chorassanicum*, 2 were *C. cunneatum*, 7 were *C. echinospermum*, 24 were *C. judaicum*, 21 were *C. pinnatifidum*, 21 were *C. reticulatum* and 2 were *C. yamashitae*.

Seeds from all accessions were increased as part of this project and are currently stored both at the University of Saskatchewan as well as at the Plant Gene Resource Centre at Agriculture and Agri-Food Canada in Saskatoon.

Table 2. Original *Cicer* accessions

Cicer Species	World Collection[1]	Our Collection
C. bijugum	20	9
C. chorassanicum	2	1
C. cunneatum	1	1
C. echinospermum	10	7
C. judaicum	34	15
C. pinnatifidum	28	16
C. reticulatum	18	6
C. yamashitae	3	2
Total	**116**	**57**

[1]Berger *et al*. 2003

Berger *et al* (2003) pointed out that many of the chickpea accessions stored in collections do not constitute independently collected wild populations but are only selections from original sources. For example, *C. bijugum* accessions ILWC 62 to 77 are actually selections from the original population ILWC 7 (Table 1, column: selection from). In addition, many accessions in world collections are either not available for distribution due to low seed supply or have lost viability. Therefore, the actual number of

accessions present in world collections and surveyed in our study was much smaller than anticipated (Table 2).

Since world collections cover only a fraction of the potential diversity available in the wild, new collections of wild populations are urgently needed, especially considering that the state of the perennial *Cicer* collection is in even worse shape than the collection of annual wild species.

3.2. *Ascochyta* blight resistance testing

This work was undertaken in collaboration with project # 20020160-04AC 'Strategy for genetic improvement of ascochyta blight resistance in chickpea *(Cicer arietinum* L)'. *Ascochyta rabiei* screening was conducted in the greenhouse using isolate Ar 82-2001, a moderately aggressive strain, which had been collected in Saskatchewan in year 2001. For visual screening a scale from 0 to 9 was used, where 0 was considered immune (no symptoms), 1 was resistant, below 6 was moderately resistant and 9 susceptible. Results presented in Table 1 showed that 104 accessions were classified as susceptible and 6 were moderately resistant at the flowering stage. No accession was rated resistant at the flowering stage. At the seedling stage, 18 accessions were rated as resistant to moderately resistant. This is in contrast to many reports in the literature (see Table 1, column: *Ascochyta* rating literature) where considerably higher resistance levels were reported. For example Collard *et al* (2001) found that *C. bijugum* accessions ILWC 7, 76 and 241 had resistance scores of 2.0 – 2.1. Yet in our screening tests, these accessions only rated between 6 and 8.5 at the flowering stage and 5.0 to 5.7 at the seedling stage, indicating that ascochyta strains in Saskatchewan are more aggressive than in other parts of the world.

It should be pointed out that wild collections consist of heterogeneous populations and that considerable variation for ascochyta blight resistance was found **within** accessions with some plants having resistant ratings of 2 (e.g. *C. judaicum* ILWC 165) at the seedling stage. These plants were harvested and maintained separately. Hence, research was focused on these accessions in order to transfer new sources of blight resistance to the cultigen.

3.3. Crossing

In 2005, a total of 4559 crosses were attempted, 1880 involving *C. bijugum* ILWC 260 and 2679 involving *C. judaicum* accessions ICC 17196, ILWC 164 and 165 (Table 3). From January to May 2006, 1574 crosses were attempted, mostly with selected accessions from *C. judaicum* ILWC 165 (Table 4).

Table 3. Crossing data from 2005

C. arietinum Genotype	Wild *Cicer* Species	Acession No.	# of Crosses	# of Ovules rescued[1]	% Ovules per Cross
CDC Diva	*C. bijugum*	ILWC260	11	0	0
Macarena	*C. bijugum*	ILWC260	289	51	17.6
Sonali	*C. bijugum*	ILWC260	674	213	31.6
ICCV10	*C. bijugum*	ILWC260	837	175	20.9
97IND-112	*C. bijugum*	ILWC260	26	0	0
ICC12512	*C. bijugum*	ILWC260	43	4	9.3
CDC Diva	*C. judaicum*	ICC17196	29	0	0
CDC Xena	*C. judaicum*	ICC17196	21	3	14.3
Macarena	*C. judaicum*	ICC17196	255	68	26.7
Sonali	*C. judaicum*	ICC17196	659	199	30.2
ICCV10	*C. judaicum*	ICC17196	746	176	23.6
97IND-112	*C. judaicum*	ICC17196	25	0	0
ICC12512	*C. judaicum*	ICC17196	48	7	14.6
Macarena	*C. judaicum*	ILWC165	80	35	43.8
Sonali	*C. judaicum*	ILWC165	273	132	48.4
ICCV10	*C. judaicum*	ILWC165	260	117	45.0
ICCV2	*C. judaicum*	ILWC165	49	23	46.9
KAK-2	*C. judaicum*	ILWC165	33	17	51.5
Sonali	*C. judaicum*	ILWC164	76	16	21.1
ICCV10	*C. judaicum*	ILWC164	100	54	54.0
KAK-2	*C. judaicum*	ILWC164	25	11	44.4

[1]Only ovules at least 2 mm in size were counted and rescued

Table 4. Crossing data from 2006

D. arietinum genotype	Wild *Cicer* Species	Acession No.	# of Crosses	# of Ovules rescued[1]	% Ovules per Cross
ICCV2	*C. bijugum*	ILWC260	30	12	40.0
KAK-2	*C. bijugum*	ILWC260	38	16	42.0
ICCV2	*C. bijugum*	ILWC69	30	29	96.0
KAK-2	*C. bijugum*	ILWC69	51	36	70.6
ICCV2	*C. judaicum*	ILWC164	95	43	45.3
KAK-2	*C. judaicum*	ILWC164	90	44	49.0
ICCV10	*C. judaicum*	ILWC165	564	348	61.7
KAK-2	*C. judaicum*	ILWC165	676	368	54.4

[1]Only ovules at least 2 mm in size were counted and rescued

3.3.1. Parents

Since genotype can have a major effect on hybridization success, a collaborative effort was undertaken to identify superior parents. Dr. Clarke (CLIMA, Australia) found two Australian cultivars, Macarena and Sonali (WACPE 2075), were better parents with respect to number and size of hybrid ovules/embryos obtained. Dr. Mallikarjuna (ICRISAT, India) identified ICCV 10, ICCV 2 and KAK-2 as superior breeding lines in her crossing experiments. Our experiments showed that Canadian genotypes CDC Cabri, Diva or Xena were not equivalent or better than the Australian or Indian parents.

An experiment was conducted comparing hybrid ovule size from crosses of ICCV-10, ICCV-2 or KAK-2 with *C. judaicum* ILWC 165. Hybrid ovules were rescued 7 days after pollination. Crosses with ICCV-10 or Canadian genotypes generally resulted in ovules of 3 mm or less at the time of rescue (Fig. 1). In contrast, ovules from crosses involving ICCV-2 or KAK-2 were often 4 mm in size. This result is of particular importance because ovule size and embryo size as well as germination rates are closely correlated as shown in Figs. 2 and 3 in section 3.3.3.D.

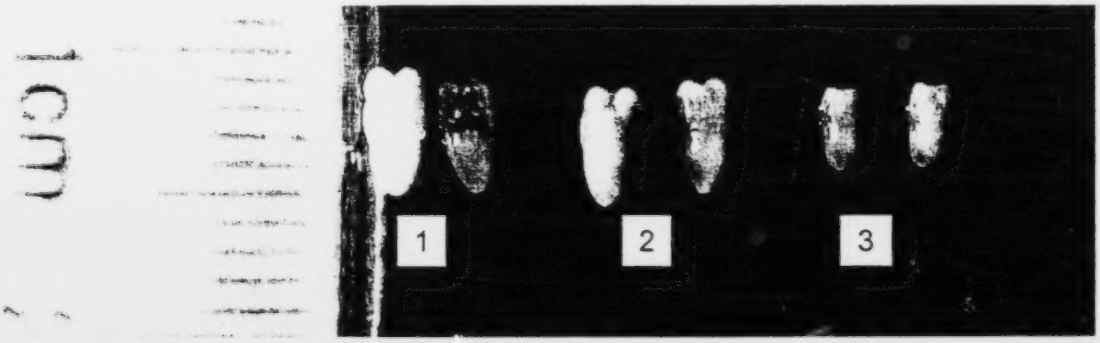

Fig. 1. 7-day-old hybrid ovule size variations from crosses of *C. arietinum* x *C. judaicum* ILWC 165. Female parents were KAK-2 (1), ICCV-2 (2) and ICCV-10 (3).

3.3.2. Bridge crossing

Since one of the milestones for year 3 was to use bridge species if wild accessions could not be directly crossed with the cultivated species, crosses were also made between *C. arietinum* and selected wild species. *C. echinospermum* was chosen because it belongs to the secondary gene pool and is fairly readily crossed with *C. arietinum* although a reciprocal translocation can cause sterility problems (Ladizinsky and Adler 1976). *C. pinnatifidum* was chosen because it belongs to the tertiary gene pool and a protocol for embryo rescue is available (Mallikarjuna 1999).

The following bridge crosses were made:	Crossed with:
C. arietinum x *C. echinospermum*	F1 x *C. judaicum* F1 x *C. bijugum*
C. arietinum x *C. pinnatifidum*	F1 x *C. judaicum*
C. pinnatifidum x *C. judaicum*	F1 x *C. arietinum*

Hybrids obtained from the first two crosses were subsequently crossed with *C. judaicum* or *C. bijugum* ILWC 260. However, no hybrids could be obtained using these bridge species.

In case of the third cross (above) between *C. pinnatifidum* and *C. judaicum*, resulting hybrids had viable pollen in their anthers (Photo 1) but no seed set could be obtained, even though they flowered profusely indicating that some kind of fertilization barrier is present in this cross. Back-crossing with *C. arietinum* also failed. These results suggest that *C. judaicum* is more distantly related to the cultigen than either *C. bijugum* or *C. pinnatifidum*.

3.3.3. *In vitro* culture technique

A. Growth regulator application

Keeping the hybrid ovules/embryos on the parent plant as long as possible increases the likelihood that embryos develop to the heart-shaped stage. This is of major importance since the embryos are more likely to survive if they reach this stage. For this purpose, a daily application of a growth regulator mix was found to be useful. However, hybrids obtained between *C. arietinum* x *C. bijugum* using this method could not be rooted (see chapter 3.6). One cause for the lack of rooting could have been the interference of the daily growth regulator application with proper root initiation in the embryo.

The following solutions were applied:

(1) 8 mg/l GA_3 + 1 mg/l NAA + 1 mg/l kinetin
(2) 8 mg/l GA_3 + 1 mg/l NAA + 1 mg/l kinetin + 10 g/l glucose

Cotton was wrapped around the abscission point between flower and parent plant and then soaked with the first solution. The second solution was only applied once to the base of the pistil at the time of crossing.

An experiment was conducted to determine if the frequency of growth regulator application (solution 1) could be changed:

(1) One time application at the time of crossing
(2) One application daily for the first three days
(3) Control: daily application until rescue (8 -12 days)

Results showed that embryos aborted if the growth regulator mix was not applied daily and embryos were very rarely viable long enough to allow recovery.

B. Pollen pre-treatment

Usually fresh pollen was used to pollinate pistils. However, in some species using pre-germinated pollen for fertilization decreased the time needed for fertilization and thus improved seed set.

An experiment was conducted to determine if the application of pre-germinated pollen would result in faster fertilization and subsequently improved embryo development using either *C. bijugum* or *C. judaicum* pollen.

Testing a variety of solutions showed that the following mixture facilitated pollen germination:

	per Liter
Boric acid	400 mg
Calcium nitrate	600 mg
Magnesium sulfate	400 mg
Potassium nitrate	200 mg
pH 8.5 – 8.8	
PEG	1 ml
C. bijugum	20% sucrose
C. judaicum	10% sucrose

Results showed that pre-germinated pollen did not speed up the fertilization process, and thus had no advantage over non-germinated, fresh pollen.

C. Ovule pre-treatment

Ovules release phenolics into the medium and thus causes browning. Phenolics are toxic to developing embryos. Ovule browning is especially a problem in crosses involving *C. bijugum*.

The effect of three treatments on ovule browning were tested:

- 5, 10, 15 or 30 minute rinse with ascorbic acid (100 mg/l)
- adding 100 mg/l PVP to the medium
- light versus dark incubation

The most effective treatment was to incorporate 100 mg/l PVP in the medium for rescue of *C. arietinum* x *C. bijugum* ovules.

D. Medium improvements

Hybrid crosses between *C. arietinum* x *C. bijugum* aborted about 10 -12 days after pollination with some embryos having reached the early heart-shaped stage (Fig. 2). In contrast, crosses involving *C. judaicum* aborted earlier (about 8 DAP) and thus hybrids were only at the pro-embryo stage. Ahmad and Slinkard (2003) reported that *C. arietinum* x *C. judaicum* hybrids consisted only of 5 cells at this stage. Our Australian colleague, Dr. H. Clarke, conducted an experiment to determine the percentage of embryo germination in relation to ovule age using selfed *C. arietinum* ovules (Fig. 2).

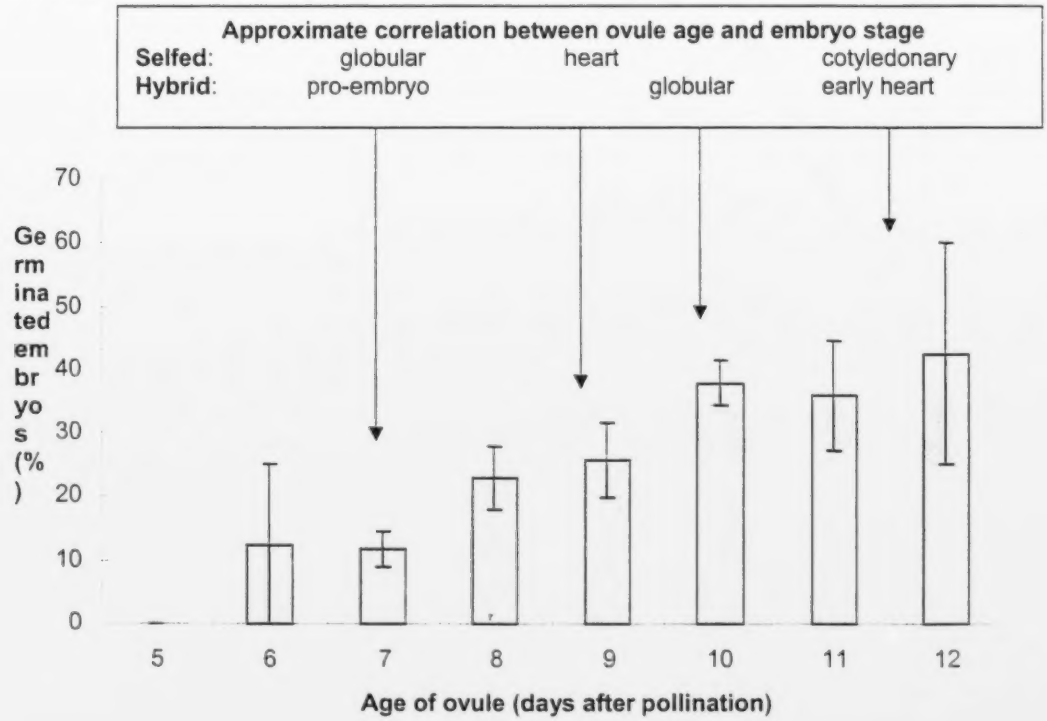

Fig. 2. Percentage embryo germination in relation to ovule age (Source: Clarke *et al* 2006)

The germination rate of 6 or 7-day old ovules with embryos at the globular stage was only about 10%. Germination rates markedly increased when the embryo reached the heart-shaped stage (about day 9) and the cotyledonary stage (about day 12).

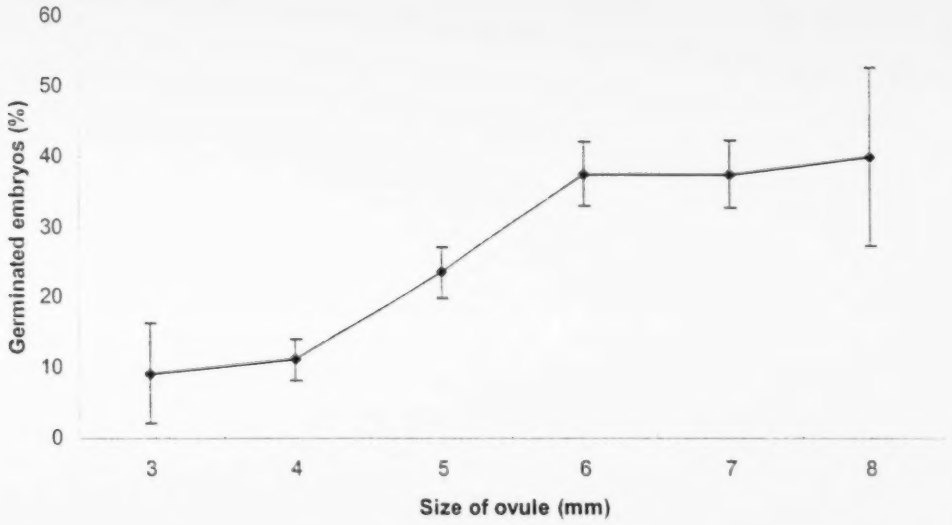

Fig. 3. Percentage embryo germination in relation to ovule size using selfed *C. arietinum* genotypes ovules (Source: Clarke *et al* 2006)

Fig. 3 shows the relationship between ovule size and embryo germination using selfed *C. arietinum* ovules. A 3 to 4 mm ovule containing a selfed embryo at the globular stage has only a 10% likelihood to germinate. Once the embryo has reached the heart-shaped stage (ovule size is about 6 mm, on day 9), the germination rate increases to 40%. This is in sharp contrast to hybrid ovules and embryos which developed considerably slower. Hence, keeping the hybrid embryos on the parent plant as long as possible was a major goal of this project (see A. Growth regulator application). In addition, the younger the embryo the more complex the medium requirements and thus developing a more complex medium for these embryos was another major goal of this project.

Using 7-day old selfed ovules a medium was developed for rescue of *C. arietinum x C. bijugum* hybrids (see Progress Report February 2005). However, the medium developed for *C. arietinum* x *C. judaicum* crosses did not support hybrid embryo growth. Seven-day old selfed ovules contain embryos at the globular stage, whereas hybrid embryos were still in the pro-embryo stage. Embryos in selfed ovules reached this stage about 5 days after pollination. Fig. 2 shows that embryo germination could not be obtained from 5-day old ovules (% germinated embryos = nil).

A major experiment was undertaken to develop a medium for embryo rescue using 5 day-old selfed ovules from *C. judaicum*. More than 20 different media and modifications were tested. The composition of the more responsive media is listed in Table 5. The

experiment consisted of 18 plates per medium with ten ovules per plate, i.e. 180 ovules per medium. Fig. 4 shows the means and standard error for these 6 media. On average, 70% of 5-day old ovules resulted in embryo germination and subsequent plantlet formation (with root and shoot) using medium RM (difference between RM-1, -2 and -3 was not significant $LSD_{0.05}$). This is a major improvement considering that previously, no germination could be obtained from 5-day-old selfed ovules (Fig. 2).

Fig. 4. Average number of embryos per plate for 6 different media (1 = L2-D14, 2 = L2-D33, 3= FNN, 4= RM-1, 5= RM-2, 6= RM-3) $LSD_{0.05}$ = 1.96

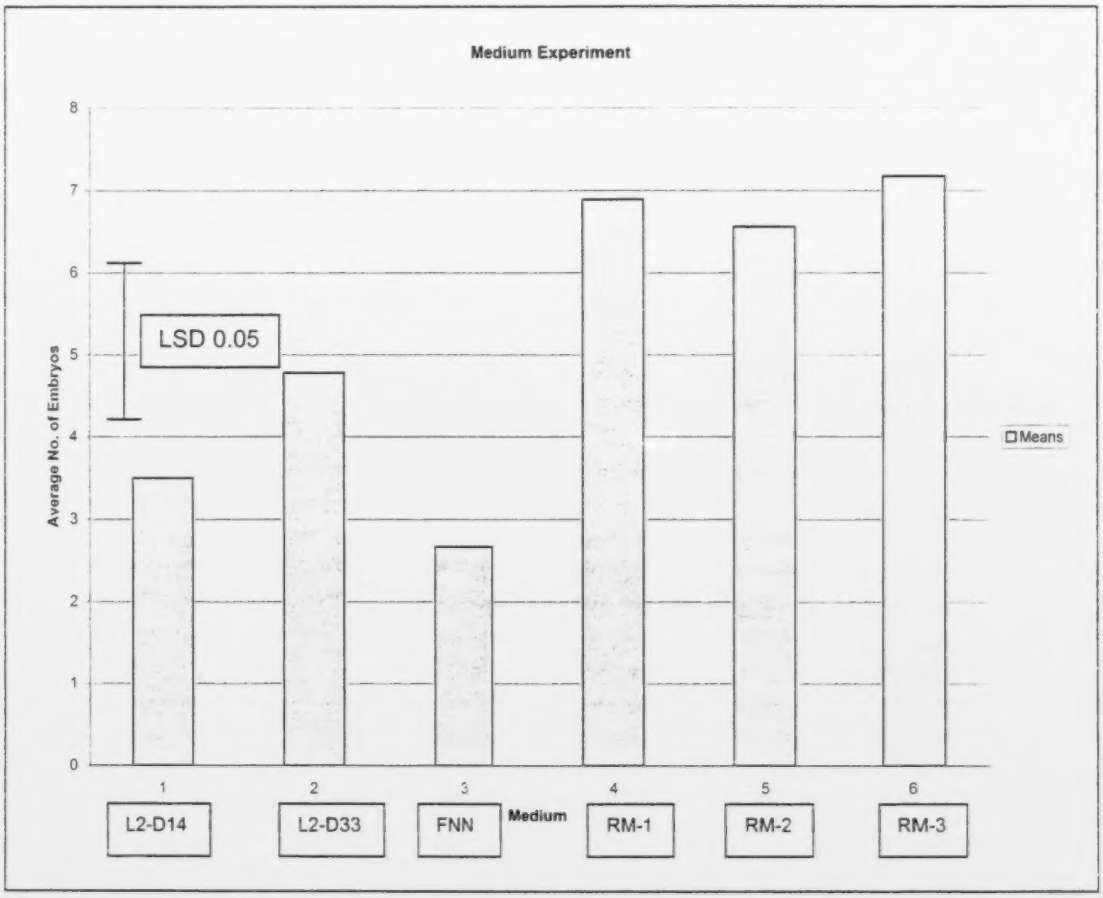

Table 5. Medium composition for rescue of 5-day old *C. judaicum* ovules

Macro in mg/l	L2-D14	L2-D33	FNN	RM-1	RM-2	RM-3
NH_4NO_3	1,000			250	250	250
KNO_3	1,000	2,820	2,820	2,500	2,500	2,500
$CaCl_2 \cdot 2H_2O$	600	600	290	440	440	440
$(NH_4)_2SO_4$	-	460	460	-	-	-
$MgSO_4 \cdot 7H_2O$	435	370	370	370	370	370
KH_2PO_4	325	190	190	170	170	170
$NaH_2PO_4 \cdot H_2O$	85			-	-	-
Micro in mg/l						
KI	1.00	1.00	1.00	1.00	1.00	1.00
H_3BO_3	5.00	5.00	6.20	6.20	6.20	6.20
$MnSO_4 \cdot H_2O$	15.00	15.00	16.90	16.90	16.90	16.90
$ZnSO_4 \cdot 7 H_2O$	5.00	5.00	8.60	8.60	8.60	8.60
$Na_2MoO_4 \cdot H_2O$	0.40	0.40	0.25	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.10	0.10	0.025	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$	0.10	0.10	0.025	0.025	0.025	0.025
NaFeEDTA	27.85	27.85	27.85	27.85	27.85	27.85
Vitamins in mg/l						
Myo-Inositol	250.00	250.00	250.00	250.00	1000.00	1000.00
Pyridoxine HCl	0.50	0.50	1.00	1.00	1.00	1.00
Thiamine HCl	2.00	2.00	10.00	10.00	10.00	10.00
Nicotinic acid	1.00	1.00	1.00	1.00	1.00	1.00
Carbohydrates						
Sucrose g/l	60.00	60.00	60.00	60.00	60.00	60.00
Acacia mg/l						100.00
Hormones						
BAP	1.00	1.00	1.00	1.00	1.00	1.00
IAA	0.25	0.25	0.25	0.25	0.25	0.25
Amino Acids						
Glutamine	500	500		800	800	800
Asparagine	250	250	890	1200	1200	1200
Arginine	200	200				
Leucine	100	100				
Lysine	100	100				
Phenylalanine	100	100				
Serine	100	100				
Casein hydro.	200	200				
Phytagel	2.5	2.5	2.5	2.25	2.25	2.25
pH	5.70	5.70	5.70	5.70	5.70	5.70

RM = Rao and De 1987

3.4. Hybrids

The following (putative) hybrids were obtained in this project:

(1) ICCV10 x *C. bijugum* ILWC 260 (D786) (Photo 2a)
(2) Sonali x *C. bijugum* ILWC 260 (H 565)
(3) Macarena x *C. bijugum* LIWC 260 (L350)
(4) ICCV 10 x *C. bijugum* LIWC 260 (M25)
(5) Sonali x *C. judaicum* ILWC 165 (M567)
(6) Sonali x *C. judaicum* ILWC 165 (M568)
(7) Macarena x *C. judaicum* ICC 17196 (I327)
(8) Macarena x *C. judaicum* ICC 17196 (K830) (Photo 2b)

3.4.1. Authentication of hybrids

Several putative hybrids were recovered from this project. Hybrid status was confirmed in 2 hybrids using SSR markers (Fig 5), ICCV10 x *C. bijugum* ILWC 260 (D786) and Sonali x *C. bijugum* ILWC 260 (H565) (for details see report from project # 20020160-04AC).

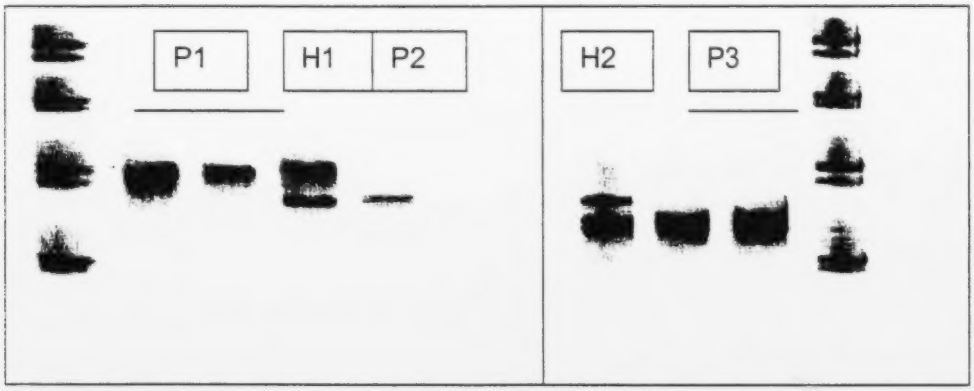

Fig. 5 SSR markers for parent 1 (ICCV10), parent 2 (*C. bijugum* ILWC 260) and parent 3 (Sonali) as well as hybrid 1 and 2.

Hybrid status has not yet been confirmed for hybrids 3 – 8 (above) because they are only poorly growing in culture. Putative hybrids 7 and 8 died in tissue culture after a short period of shoot growth.

3.4.2. Improvements to shoot multiplication medium

Generally, hybrids were slow to germinate and grow in tissue culture compared to either parent. In addition, published media did not support root or shoot growth and needed improvement especially after prolonged growth in tissue culture.

Initially, hybrid shoots were propagated successfully using Heather Clarke's micropropagation medium consisting of MS macro and micro-nutrients, B5 vitamins, 30 g/l sucrose, 0.1 mg/l BAP and 0.01 mg/l NAA (control). However, after prolonged culture, hybrid shoots show signs of vitrification, lack of chlorophyll development as well as lack of growth.

An experiment was conducted trying to improve shoot growth in culture. Seven different media were tested in comparison to the control. The composition of the different shoot growth media is given in Table 6. None of the media tested to date has improved hybrid shoots growing in culture for prolonged periods of time.

Table 6. Composition of shoot growth media

Code	Macro	Micro	Vitam	BAP	Zea	Sucr	Agar
CP-S1	½ MS	½ MS	½ MS		1	30	8
CP-S2	½ MS (750 Ca)	½ MS	½ MS		1	30	8
CP-S3	½ MS (500 Ca)	B5	B5		1	30	8
CP-S4	½ MS (500 Ca)	B5	B5	1		30	8
CP-S5	½ MS (500 Ca)	B5	B5	2		30	8
CP-S6	½ MS (500 Ca)	B5	B5	1	1	30	8
CP-S7	½ MS (500 Ca)[1]	B5	B5	1		30	8

*Add 500 mg/L calcium instead of the amount in ½ MS
[1] CP-S7: add 80 mg/L adenine sulfate. It has some cytokinin like effect.

3.4.3. Improvements to rooting protocol of hybrids

Rooting is frequently a problem in tissue culture, especially with pulse crops. Lack of root development was a major problem in obtaining interspecific chickpea hybrids and published *C. arietinum* rooting media were not successful in rooting hybrid material.

Hybrid shoots from ICCV10 x *C. bijugum* ILWC 260 (D786), Macarena x *C. bijugum* ILWC 260 (L350) and *C. pinnatifidum* x *C. judaicum* (L496, L591) were multiplied and used for these experiments. The following procedures were tested.

(1) Grafting

Micrografting shoots onto root stock was developed by Gulati *et al* (2001) for lentil and had a 96% success rate. This procedure was successfully used in our lab for rooting lentil interspecific hybrids.

Seeds from *C. arietinum* breeding line Sonali or *C. bijugum* ILWC 260 were sterilized and aseptically germinated in Magenta boxes. Once shoots and roots were developed, the shoots were decapitated, spliced and the hybrid shoots inserted making sure that the vascular tissues were in contact with each other. About 30 shoots from hybrid ICCV10 x *C. bijugum* ILWC 260 (D786) were grafted. None of our chickpea grafts was successful probably due to poor vigor of the hybrid shoots.

(2) Fratini and Ruiz Method

Fratini and Ruiz (2003) developed a rooting procedure for lentil and chickpea based on explant polarity. This method required that nodal segments were placed in an inverted orientation into the medium resulting in 95% rooting. The authors speculated that low oxygen in the medium caused the poor rooting response in legumes.

About 30 hybrid shoots (D786) were placed in an inverted orientation into culture tubes. None of the hybrids rooted even after 2 months in culture and the hybrid shoots eventually all died.

(3) Newell *et al* Method

Newell *et al* (2005) developed a rooting protocol for 28 Australian species using a sterile propagation mixture that had improved aerobic texture. The mix consisted of sphagnum peat, coarse river sand and Perlite®.

This mixture was prepared according to instructions, put into Magenta boxes and sterilized. Sterile hybrid shoots (D786) were first exposed to 40 µM IBA for 7 days and then placed into the Magenta boxes. Results showed that none of the hybrid cuttings produced roots in this medium. This mixture was subsequently tested on *C. pinnatifidum* x *C. judaicum* hybrids. A small percentage of these hybrids rooted in this medium probably because cuttings from these hybrids were more vigorous than the *C. arietinum* x *C. bijugum* hybrids.

(4) Rooting Medium

Initially, rooting was attempted using published protocols for chickpea as well as a medium (Table 6) developed by our collaborator, Dr. H. Clarke (CLIMA, Australia). Unfortunately, none of our hybrids produced roots on this medium. Subsequently, this medium as well as published protocols were modified and tested using either filter-

paper bridges, phytagel-solidified medium or Newell *et al* (2005) propagation mixture. The following factors were tested:

- Full or half strength MS macro- or micro-nutrients
- Auxins: IAA, IBA, NAA, 2,4-D, or picloram (alone or in combination)
- Concentrations tested: 1, 2, 3, 4, 5, 6, 7, 8, and 20 mg/L
- Other additions: 0.9 mg or 88.2 mg/l putrescine or 0.15 or 145.3 mg/l spermidine (Tang *et al* 2005), charcoal, calcium, calcium pantothenate, or arginine, 7 different amino acids as well as casein hydrolysate

Using ICCV10 x *C. bijugum* ILWC 260 hybrid (D786) shoots only 1 shoot each was rooted on CP-R4 and CP-R7. However, subsequent growth was poor and hybrids died. These media consisted of ½ MS macro- and micro-nutrients, 500 mg/l $CaCl_2$, B5 vitamins, 2 mg/l IBA and 50 mg/l arginine (CP-R4).

Another experiment was conducted using Macarena x *C. bijugum* 260 hybrid shoots (L350). The media tested had added amino acids as well as 5 or 10 mg/L NAA and different substrates. None of the shoots produced roots on L2-D30 or L2-D31 medium or any of the modifications regardless if Phytagel®, Newell *et al* mixture, or filter–paper bridges were used. In contrast, *C. pinnatifidum x C. judaicum* ICC 17196 hybrid (L496) shoots survived on both media although the percentage of rooting was low (7-8%).

Developing a rooting protocol was hampered by:

(1) Low number of hybrid shoots available for testing
(2) Rooting media developed with non-hybrid material was not suitable for hybrid shoots
(3) Lack of vigour of the hybrid plant material.

3.5. Barriers to interspecific hybridization

Since hybrids did not flower *in vitro* and could not be transferred to soil, the following milestones could not be investigated:

- Pollen fertility assessment and cytogenetic characterization of hybrids
- Selfing or back-crossing of hybrids and determination of problem areas
- Transfer of hybrid material and/or selfed or back-crossed hybrids to the CDC pulse breeding program for cultivar improvement

In order to determine the specific barriers to interspecific hybridization with *C. judaicum*, a range of tests were conducted as follows.

3.5.1. Pollen viability

Tests showed that pollen viability was good in all our parent material. *C. judaicum* had approximately 8.800 pollen microspores/ml where *C. bijugum* had 5,050 microspores/ml indicating that poor pollen quality was not the cause for the low number of hybrids obtained.

C. pinnatifidum x C. judaicum crosses were made in order to use the hybrids for bridge crossing. These plants were transferred to soil and flowered profusely, yet no seed set could be obtained. Pollen viability tests also showed viable pollen (photo #1) in this cross.

3.5.2. Pollen germination on pistils

Pollen germination was examined using KAK-2 x *C. judaicum* ILWC 165 crosses. Fluorescent staining showed that *C. judaicum* pollen germinated on the *C. arietinum* pistil. However, pollen tube growth through the style was slow, progressing about half-way through the pistil after 24 hours (Photo 3a) and it took about 48 hours (Photo 3b) until the ovules were reached. Ahmad *et al* (1988) estimated the time to fertilization to be about 27.4 ± 8.8 hours for *C. arietinum* x *C. judaicum* (ICCW 33, 35 and 36) crosses in their experiments. Stamigna *et al* (2000) reported that in their experiments hybrid zygote formation took place within 48 hours after pollination. Intra-species variation for specific cross-compatibilities is not uncommon.

3.5.3. Zygote formation

Microscopic examination showed that pollen tubes grew through the style and reached the ovules after about 48 hours i.e. fertilization occurred. These results are similar to studies by Stamigna *et al* (2000).

3.5.4. Embryo growth and plant regeneration

Dissection of ovules showed that pro-embryos were present in many *C. arietinum* x *C. judaicum* crosses (Photos 4 a and b). However, these embryos rarely progressed to heart-shaped and cotyledonary stages. In addition, the maternal tissue surrounding the embryo often seemed to be growing into the embryo cavity indicating abnormalities between endosperm (maternal) and hybrid embryo tissue.

Ahmad and Slinkard (2003) reported that *C. arietinum* x *C. judaicum* crosses died prior to day 10 whereas the endosperm was still alive on day 12. Stamigna *et al* (2000) reported hybrids had reached the pro-embryo stage 3 DAP and the globular stage on day 6-7. At this time, endosperm degeneration began.

4. Conclusions

Wild Cicer species are sources for many desirable traits necessary for the improvement of the cultivated chickpea. Research in recent years has succeeded in using *C. echinospermum*, *C. reticulatum* and *C. pinnatifidum* for the genetic improvement of the cultigen. Yet accessing the 5 remaining annual species has proven difficult. Confirmed hybrids with *C. bijugum* were obtained in this project. Thus far, using these hybrids in the crossing program has been hampered by the lack of rooting and subsequent adaptation to normal growing conditions. Cross-ability barriers clearly operate post-fertilization and most probably through genetic disharmony between maternal and paternal genotype.

With the expiration of this project, Canadian research in the use of wild related *Cicer* species for the improvement of ascochyta blight resistance in chickpea will cease, but research will continue in Australia (CLIMA) and India (ICRISAT).

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E. PERSONNEL

Dr. Lulsdorf was the research scientist and Tim Dament was the technichian working on this project. Tana Karchewski worked on a part time basis on this project.

F. EQUIPMENT

No equipment was purchased for this project.

G. PROJECT DEVELOPED MATERIALS

Presentations and Conference Papers:
Lulsdorf, M; H. Clarke; N. Mallikarjuna; B. Tar'an 2005. Finding Solutions for Interspecific Hybridization Problems in Chickpea. IFLRC-IV conference, New Delhi, India, October 2005

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Poster:
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H. PROJECT PHOTOS

Photos are attached in a separate file.

I. ACKNOWLEDGEMENTS

We appreciate the donation of wild *Cicer* accessions by Dr. Faiz Ahmad (Brandon, Manitoba), CLIMA-ATC (Australia), USDA-USR (USA), ICRISAT (India) and ICARDA (Syria). Financial support from Saskatchewan Pulse Growers and Saskatchewan Agriculture & Agrifood is also gratefully acknowledged.

H. Project Photos

Photo 1. Pollen from *C. pinnatifidum* x *C. judaicum* hybrids stained with acetocarmine

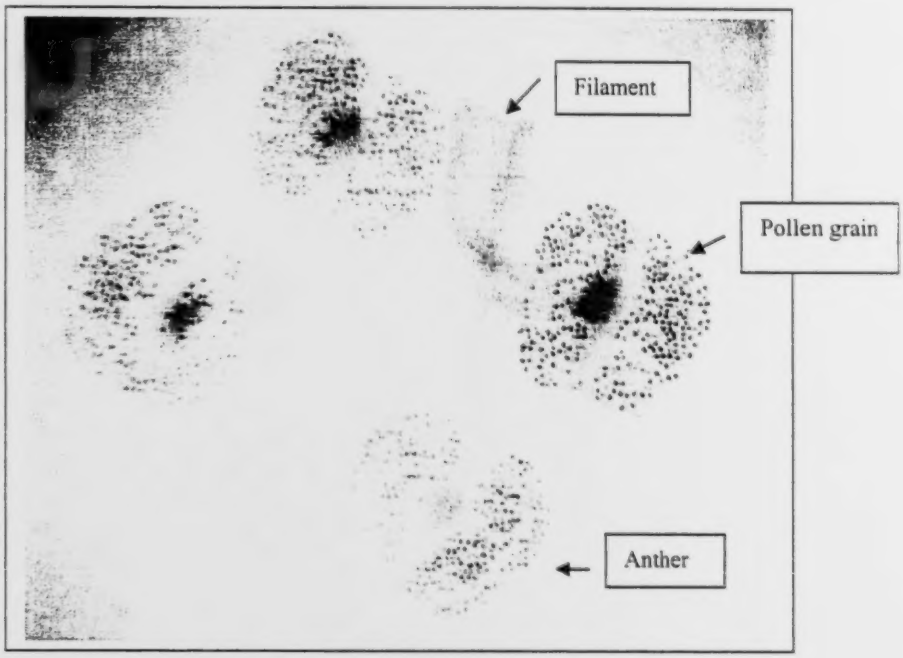

Photo 2.a. Hybrid shoots from cross ICCV10 x *C. bijugum* ILWC 260 (D786) and
2. b. Hybrid plantlet from Macarena x *C. judaicum* ICC 17196

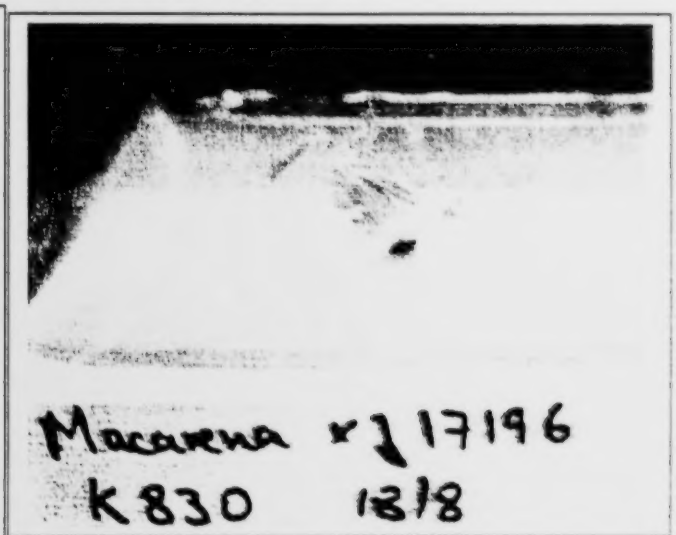

Photo 3a, b. Pollen tube growth through style after 24 (a) and 48 (b) hours

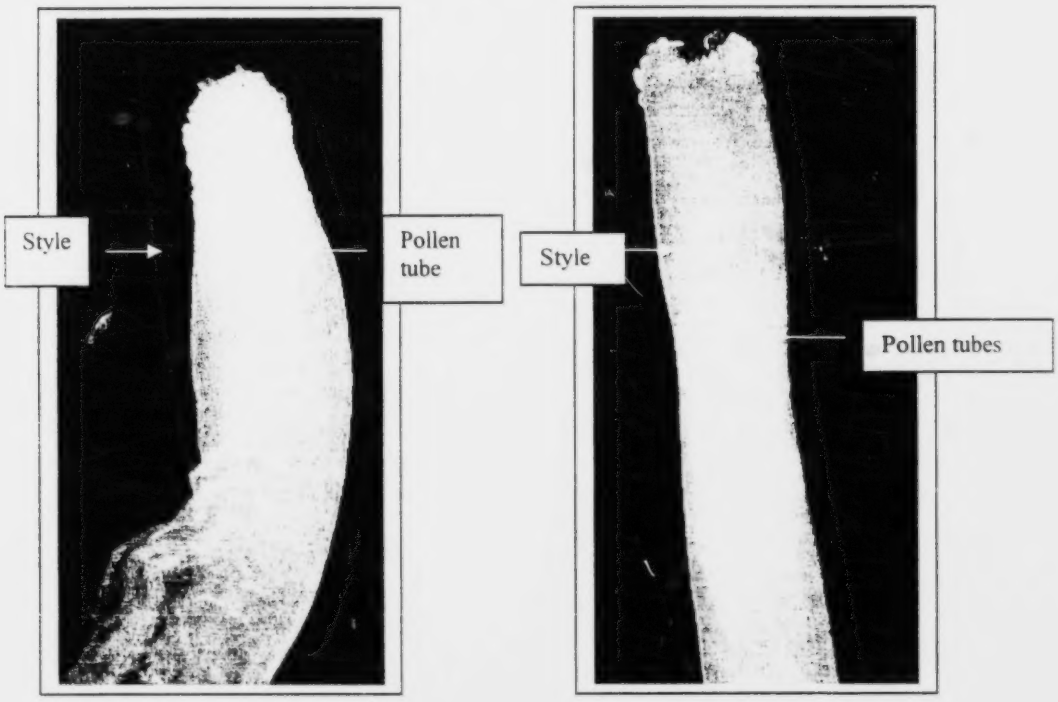

Photo 4. Hybrid embryos from *C. arietinum x C. judaicum* crosses